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Glutamate and Dynorphin Release From a Subcellular Fraction Enriched in Hippocampal Mossy Fiber Synaptosomes[1]

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TERRIAN, D. M., D. JOHNSTON, B. J. CLAIBORNE, R. ANSAH-YIADOM, W. J. STRITTMATTER AND M. A. REA. *Glutamate and dynorphin release from a subcellular fraction enriched in hippocampal mossy fiber synaptosomes*. BRAIN RES BULL 21(3) 343–351, 1988.—A procedure is described for the isolation of intact hippocampal mossy fiber synaptosomes. Electron microscopic examination revealed numerous synaptosomal profiles which are clearly of mossy fiber origin, indicated by their large size (2–6 μm diameter) and characteristic morphology. Furthermore, this fraction is enriched in zinc and dynorphin B which appear to be concentrated in mossy fiber terminals in vivo. Synaptosomes isolated by this procedure accumulated 2-deoxyglucose and retained 88% of total lactate dehydrogenase activity after incubation at 30°C for 60 minutes, indicating a high degree of membrane integrity. Oxygen consumption was stimulated 4-fold by veratridine (0.1 mM) and inhibited 90% by ouabain (1 mM), suggesting that synaptosomal metabolism remained tightly coupled to ouabain-sensitive ATPase activity. Potassium-stimulated (45 mM) release of dynorphin B was completely dependent upon the presence of extrasynaptosomal calcium, while only 30% of the evoked release of glutamate was calcium-dependent. D-aspartate, which exchanges glutamate out of the cytoplasmic pool, virtually eliminated the calcium-independent component of glutamate release. This synaptosomal preparation will be useful in identifying the factors that modulate the release of amino acid and opioid neurotransmitters from hippocampal nerve terminals and in the investigation of their presynaptic mechanisms of action.

Hippocampus Mossy fiber expansions Synaptosomes Glutamate Dynorphin Peptides Opioids Release Calcium

HIPPOCAMPAL mossy fiber synapses, which arise from the axons of the dentate granule cells and terminate mostly on the pyramidal neurons of the CA3 subfield, have a number of relatively unique features. The synaptic boutons are quite large (4–8 μm) (2), and high concentrations of zinc (9,11) and several of the opioid peptides (20,26) appear to be localized in the presynaptic endings. The mossy fibers form a narrow band in the proximal portion of the apical dendrites (stratum lucidum) of the CA3 region, and the mossy fiber synapses are estimated to be within 6% of a length constant from the somata of the pyramidal neurons (23). This relatively short electrotonic distance from the subsynaptic membrane to the pyramidal cell soma has facilitated the detailed biophysical analysis of the conductance properties of this excitatory synapse under normal conditions and during long-term synaptic potentiation (LTP) (4, 6, 18). The mossy fiber synapses are the only synapses in the mammalian central nervous system for which such information is available.

Comparatively less neurochemical information is available for the mossy fiber synapses. Glutamic acid is considered a likely candidate for a neurotransmitter at this synapse based on immunohistochemical evidence (1, 32, 40), the identification of high affinity uptake sites (33), the release of endogenous glutamate in response to electrical stimulation of the mossy fibers (10) and the response of CA3 neurons to exogenously applied glutamate (30). Other probable neurotransmitter candidates include the prodynorphin-derived peptides, which are densely concentrated in the hippocampal mossy fiber system (20,26) and are released in a calcium-dependent manner with depolarization of hippocampal slices (7), and zinc, which is also released from the mossy fibers by a calcium-dependent mechanism (21). These data suggest

[1]The animals involved in this study were procured, maintained and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources—National Research Council.

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that there may be multiple substances acting as neurotransmitters at this synapse. The release mechanisms and the possible interactions and the functions of these different neurotransmitter substances for mossy fiber synaptic transmission and LTP, however, are unknown.

In this report we describe the morphological properties of a synaptosomal preparation enriched in hippocampal mossy fiber terminals. The preparation appears to be advantageous for biochemical investigations of this specialized synaptic terminal. We also describe features of the depolarization-induced release of endogenous glutamate and dynorphin peptides from these terminals.

METHOD

Subcellular Fractionation

Adult, male Wistar rats, weighing 150–200 g, were obtained from Charles River (Kingston, NY). Subcellular fractions were prepared from 10 to 20 rat hippocampi removed after decapitation and the tissue was manually homogenized in 9 volumes of 0.3 M sucrose using modified custom-machined stainless steel Dounce-type homogenizers with an annulus length of 0.15 cm and clearances of 0.25 mm and 0.15 mm according to the method of Hajos *et al.* (19). Magnesium ions (1 mM $MgSO_4$) were included in all solutions used in the fractionation procedure to stabilize the membranes of the larger synaptosomes (3) and 15 mM sodium N-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (Na-TES) was also included as a buffer and adjusted to pH 7.4. The homogenates were passed through a series of nylon filters (111, 70 and 52 μm mesh opening) and centrifuged at 900×g for 10 minutes at 0 to 5°C in a Sorvall RC2-B refrigerated centrifuge. The pellet was washed with an equal volume of 0.3 M sucrose and resedimented. The resulting supernatants were pooled and centrifuged at 17,000×g for 55 minutes. This procedure yielded a pellet, roughly equivalent to the crude mitochondrial (P_2) fraction of Whittaker (39) which has been shown to contain small (0.5 μm diameter) synaptosomes as well as various other subcellular particles. The larger synaptosomes sedimented with the nuclei during the initial low-speed centrifugation. This pellet was resuspended in 18% (w/v) Ficoll in 0.3 M sucrose and centrifuged at 7,500×g for 40 minutes using an SW28 rotor and Beckman L5-50B ultracentrifuge (total accumulated centrifugal effect, w^2t, equal to 175 $rad^2/sec \times 10^7$). Nuclei were sedimented under these conditions and the large synaptosomes remaining in suspension were diluted with 2 volumes of 0.3 M sucrose and centrifuged at 13,000×g for 20 minutes. The resulting supernatant was discarded and the pellet (P_3), containing the large synaptosomes, was used in all of the experiments described below unless otherwise specified. In these additional experiments the P_3 fraction was further resolved by centrifugation through 1.1 M sucrose at 53,000×g for 30 minutes to remove large fragments of medullated fibers found in P_3, the resulting pellet was designated P_4. The final pellets were resuspended in an appropriate buffer and aliquots were taken for estimates of protein concentration using the protein assay method of Lowry *et al.* (25), as modified by Peterson (28), with bovine serum albumin as the protein standard.

Electron Microscopy and Morphometric Analyses

Samples of the homogenate and the P_2, P_3 and P_4 subcellular fractions were suspended in a modified Karnovsky's fixative (1% paraformaldehyde, 1.25% glutaraldehyde, 0.072 M cacodylate buffer, 0.045 M $MgCl_2$ and 0.025% $AlCl_3$ at 4°C for 15 minutes, sedimented in microcapillary tubes and left to stand for an additional hour in the fixative at room temperature. Details of the post fixation, embedding and sectioning procedures have been described elsewhere (34). Micrographs were taken for morphometric analysis using a Hitachi HU-12A transmission electron microscope at magnifications between 7,000 and 15,000. The morphological properties of these structures were measured using a Model LGD-1 Ladd Graphical Digitizer.

Analytical Methods and Enzyme Assays

Enzyme activities were measured at 37°C, unless stated otherwise, and the methods used are indicated: lactate dehydrogenase (EC 1.1.1.27; LDH) (36); (Na^+-K^+)-ATPase (EC 3.6.1.3) (38); choline acetyltransferase (EC 2.3.1.6; ChAT) activity was determined in sonically-disrupted (Artek Model 150; 3 seconds at a setting of 30 percent) synaptosomes according to the method of Fonnum (16).

Glutaminase (EC 3.5.1.2) activity was determined as described by Kvamme *et al.* (24) using, as the incubation buffer, a Krebs-phosphate medium (pH 7.4) containing 20 mM Na_2HPO_4 (5), 10 mM glucose, 4 mM glutamine and 1 mg/ml oligomycin (Sigma Chemical Co., St. Louis, MO). Hippocampal synaptosomes (0.3 mg/ml final concentration) in 1.5 ml microfuge tubes were preincubated for 5 minutes at 37°C prior to the addition of substrate to yield a final volume of 100 μl. After an additional incubation period of 10 minutes, the reaction was terminated by the addition of 200 μl of absolute ethanol followed by centrifugation for 2 minutes in an Eppendorf microfuge. Glutamic acid produced during the initial reaction was assayed fluorometrically (17).

The percentage of total LDH activity which remained occluded during incubation of synaptosomes at 30°C was determined by the method of Dagani and Erecinska (12). Fractions analyzed for zinc were diluted to the desired protein concentration and assayed in triplicate on a Perkin-Elmer Model 5000 atomic absorption spectrophotometer. Oxygen consumption rates were determined on a YSI Model 53 oxygen monitoring system as outlined by Scott and Nicholls (31). Glutamic acid was determined using the fluorometric assay described by Graham and Aprison (17) with a Perkin-Elmer Model LS5B luminescence spectrometer to measure the oxidation of L-glutamate to alpha-ketoglutarate by glutamate dehydrogenase (EC 1.4.1.2.; GDH; Calbiochem lot no. 703238).

Uptake kinetics for [^{3}H]-2-deoxy-D-glucose (2-DG) into P_4 synaptosomes were determined using a modification of the method of Diamond and Fishman (13). Synaptosomes were suspended in a modified Elliott's medium (final concentrations: 122 mM NaCl, 3.1 mM KCl, 1.2 nM $MgSO_4$, 1.8 mM $CaCl_2$, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Na-TES; pH 7.4) containing 10 mM sodium pyruvate. Approximately 0.5 mg of P_4 protein was incubated together with [^{3}H]-2-DG (100,000 cpm; 0.005 to 1.0 mM) for 5 minutes at 25°C; controls were incubated in ice. The synaptosomes were collected on glass fiber filters (Whatman GFB) and washed three times with 4 ml of ice cold buffer. The accumulated radioactivity was determined using an LKB RackBeta liquid scintillation system (counting efficiency greater than 35%).

Dynorphin Radioimmunoassays

Dynorphin (Dyn) B and Dyn A(1–8) were determined by double antibody radioimmunoassay (RIA) according to a procedure described previously (37) using antisera, goat

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A procedure is described for the isolation of intact hippocampal mossy fiber synaptosomes. Electron microscopic examination revealed the presence of numerous synaptosomal profiles which are clearly of mossy fiber origin as indicated by their large size (2 - 6 um diameter) and characteristic morphology. Furthermore, this fraction is enriched in zinc and dynorphin B which appear to be concentrated in mossy fiber terminals in vivo. Synaptosomes isolated by this procedure accumulated 2-deoxyglucose and retained 88% of total lactic dehydrogenase activity after incubation at 30 °C for 60 minutes, indicating a high degree of membrane integrity. Oxygen consumption was stimulated 4-fold by veratradine (0.1 mM) and inhibited 90% by ouabain (1 mM), suggesting that synaptosomal metabolism remained tightly coupled to ouabain-sensitive ATPase activity. Potassium-stimulated (45 mM) release of dynorphin B was completely dependent upon the presence of extrasynaptosomal calcium, while only 30% of the evoked release of glutamate was calcium-dependent. D-aspartate, which exchanges glutamate out of the cytoplasmic pool, virtually eliminated the calcium independent component of glutamate release.

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19. Abstract
This synaptosomal preparation will be useful in identifying the factors that modulate the release of amino acid and opioid neurotransmitters from hippocampal nerve terminals and in the investigation of their presynaptic mechanisms of action.

anti-rabbit IgG and radioiodinated peptides obtained from Peninsula Labs (Belmont, CA) and synthetic peptides purchased from Sigma (St. Louis, MO). No crossreactivity (<0.2%) was observed between the Dyn B antiserum and Dyn A(1–17), A(1–13) and Dyn A(1–8), or between the Dyn A(1–8) antiserum and Dyn B, A(1–17) and A(1–13), at concentrations equal to 500 times the IC_{50} value for Dyn B or Dyn A(1–8), respectively. Serial dilutions of acidic extracts of P_3 synaptosomes yielded displacement curves for [^{125}I]-Dyn B and -Dyn A(1–8) which were parallel to those obtained with the corresponding synthetic peptides. Reversed-phase high performance liquid chromatography (7) of P_3 synaptosomal superfusates revealed that greater than 95% of Dyn B and 90% of Dyn A(1–8) immunoreactivity coeluted with their respective synthetic peptides. The limit of sensitivity of the Dyn B RIA was 7 pg and the intra- and interassay coefficients of variation were 3.6% and 12.1%, respectively. The limit of sensitivity of the Dyn A(1–8) RIA was 2.5 picograms and the intra- and interassay coefficients of variation were 3.5% and 4.8%, respectively.

Release of Glutamate and Dynorphin Peptides

Neurotransmitter release from hippocampal synaptosomes was investigated using a superfusion technique similar to that described by Flint *et al.* (15). The fritted Teflon disks at the base of polypropylene Econocolumns (Bio-Rad; Richmond, CA) were covered with glass fiber material (Whatman GFB) and 0.8 cm^3 columns of Sephadex G10 were poured. The columns were equilibrated with 10 ml of a Krebs-bicarbonate medium (final concentrations: 127 mM NaCl, 3.9 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.8 mM KH_2PO_4, 20 mM $NaHCO_3$, 11 mM D-Glucose; pH 7.4) containing 30 μg/ml bacitracin and 0.1% bovine serum albumin (KBM). The bovine serum albumin was included to prevent nonspecific binding of released peptides to the superfusion apparatus and tubing. Approximately 7 mg of synaptosomal protein suspended in KBM were applied to the top of the Sephadex G10 column. The tissue was covered with a 0.3 cm^3 blanket of Sephadex to prevent disruption of the tissue layer during media changes. A flow rate of 0.5 ml/min was maintained using a peristaltic pump. After an 8 minute washout period, sequential 4 minute fractions were collected into 10 ml polypropylene tubes. Typically, two 4 minute KBM fractions were collected prior to and following the collection of a fraction that included a 2 minute pulse of 45 mM KCl in KBM (High K^+; equimolar substitution of KCl for NaCl). Immediately after collection, each fraction was boiled for 15 minutes to destroy peptidase activity, frozen to −80°C and lyophilized overnight. Glass superfusion chambers were used, as described previously (14), in experiments conducted to examine the effects of D-aspartate on glutamate release. In these experiments, 1 minute samples were collected and superfusates were assayed for glutamic acid.

RESULTS

Electron Microscopic and Morphometric Analyses

Samples of the homogenate and of fractions P_2, P_3 and P_4 were examined with the electron microscope. As expected, the homogenate was heterogeneous, consisting of small synaptosomes (less than 2 μm in diameter), mitochondria, nuclei, medullated fibers, fragments of dendrites and red blood cells (data not shown). Importantly, it also contained numerous profiles of synaptosomes between 2 and 6 μm in diameter that were easily identifiable as mossy fiber terminals (see the Discussion section). The membranes surrounding these synaptosomes were intact, indicating that the homogenization procedure did not cause undue disruption. The P_2 fraction, prepared from the initial low-speed supernatant, was composed of mitochondria and numerous synaptosomes less than 1 μm in diameter (Fig. 1A). Only a few dendritic profiles and larger synaptosomes were encountered in this fraction. In contrast, the P_3 fraction was found to consist primarily of larger synaptosomes, again easily recognizable as mossy fiber terminals, smaller synaptic profiles (less than 2 μm in diameter) and mitochondria (Fig. 1B). Some nuclei and medullated fibers were also seen in this fraction.

In an attempt to prepare a fraction with fewer mitochondria and small synaptosomes, and without any nuclei or medullated fibers, the P_3 fraction was further resolved on a discontinuous sucrose gradient (see the Method section). The resulting fraction which sedimented through the 1.1 M sucrose layer (the P_4 fraction) was examined with the electron microscope. It consisted primarily of the larger mossy fiber synaptosomes, some smaller synaptic profiles and mitochondria. Only rarely were nuclei or medullated fibers seen. The mossy fiber synaptosomes in this fraction, however, appeared to exhibit fewer synaptic contacts and had a lower density of clear synaptic vesicles than did the mossy fiber synaptosomes in P_3. We therefore selected micrographs of 18 mossy fiber synaptosomes from each fraction for morphometric analysis, with the only selection criterion being that each profile have a long axis of at least 2 μm. As seen in Table 1, results confirmed our subjective impression that the P_4 mossy fiber terminals had fewer vesicles per square micrometer and fewer synaptic contacts per profile than did the P_3 mossy fiber synaptosomes, suggesting that the synaptosomes in the P_4 fraction were adversely affected by the hyperosmolarity of the final purification step.

Metabolic Properties

Before deciding whether to use the P_3 or the P_4 fraction for release studies, we wanted to determine whether or not the P_4 synaptosomes were metabolically active. Because the

TABLE 1

MORPHOMETRICS OF HIPPOCAMPAL SYNAPTOSOMES

Measurements	Synaptosomal Preparation P_3	P_4
Perimeter (μm)	11.12 ± 3.96	10.61 ± 4.85
Area (μm^2)	3.89 ± 2.98	4.99 ± 2.95
Circulatory index (R)*	0.39	0.56
Long axis diameter (μm)	3.9 ± 1.1	3.1 ± 1.2
Spines/profile†	2.9 ± 1.9	8.4 ± 4.1
Synaptic contacts/profile‡	8.1 ± 3.8	5.8 ± 3.9
Mitochondria/profile	4.2 ± 2.2	3.3 ± 2.4
Vesicles/μm^2	53 ± 19	18 ± 5

The morphometrics of large (diameter of greater than 2 μm) synaptosomes contained in the P_3 and P_4 fractions were analyzed by graphic digitization. Results are expressed as the means ± SD of measurements made on 18 synaptic profiles from each fraction.

*Calculated as $R = 4\pi \cdot \text{area}/\text{perimeter}^2$, according to the method of Amaral and Dent (2).

†Only dendritic spines which were at least partially encapsulated by the synaptosomal plasma membrane were counted.

‡Values include both symmetric and asymmetric synaptic contacts.

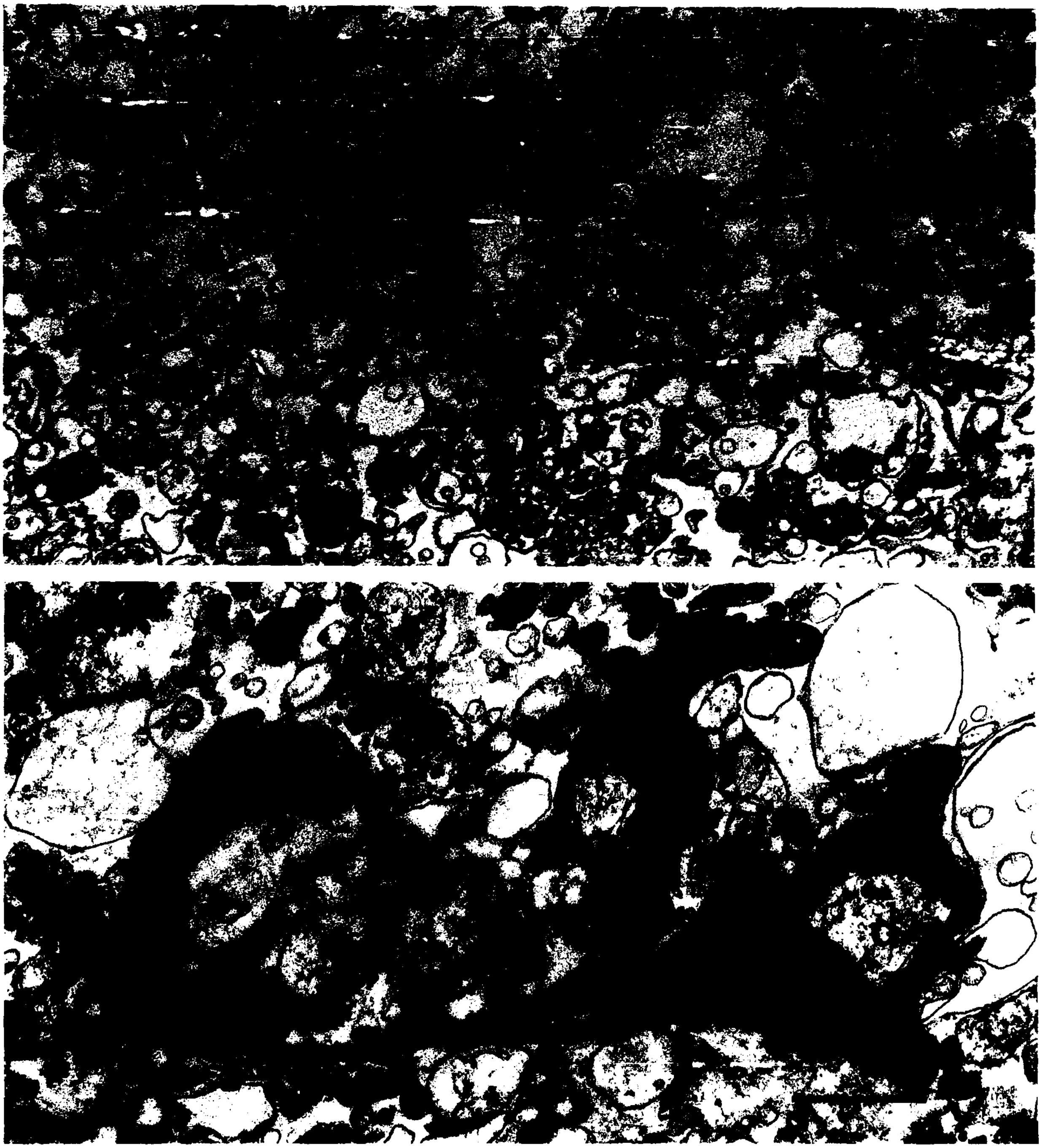

FIG. 1. Electron micrographs of subcellular fractions prepared from hippocampal tissue of the rat brain. Samples were prepared as described in the Method section. A: A cross section of the crude mitochondrial (P_2) fraction typically contained numerous small, rounded, profiles (s) surrounding mitochondria and synaptic vesicles; free mitochondria (m) and myelin fragments were also present. B: Mossy fiber synaptosomes (mf) encountered in the P_3 fraction were densely packed with synaptic vesicles and mitochondria, and could be easily identified on the basis of their size and irregular outline which were typically invaginated by two or more dendritic (d) spines with multiple asymmetric contacts (arrows). Final magnification ×21,375; scale bar = 1 μm.

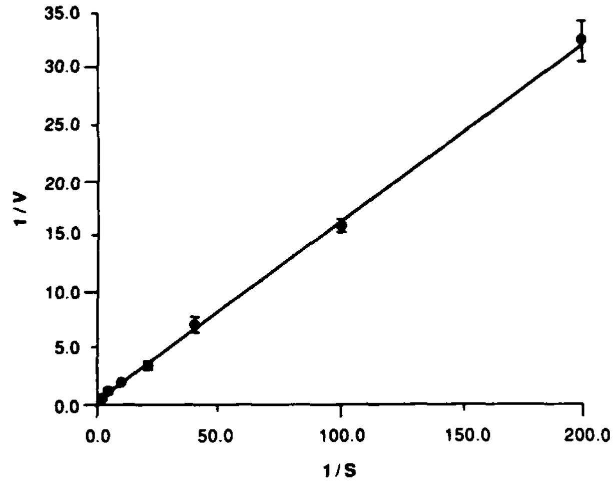

FIG. 2. Analysis of [^{3}H]-2-deoxy-D-glucose (DG) uptake, using the Lineweaver-Burk format. [^{3}H]-2-DG uptake was measured as described in the Method section. Kinetic parameters, K_t and V_{max}, were obtained by linear regression and are, respectively, 0.38 mM and 2.4 nmol/min/mg protein. The data are the means±SD of triplicate determinations in a single experiment.

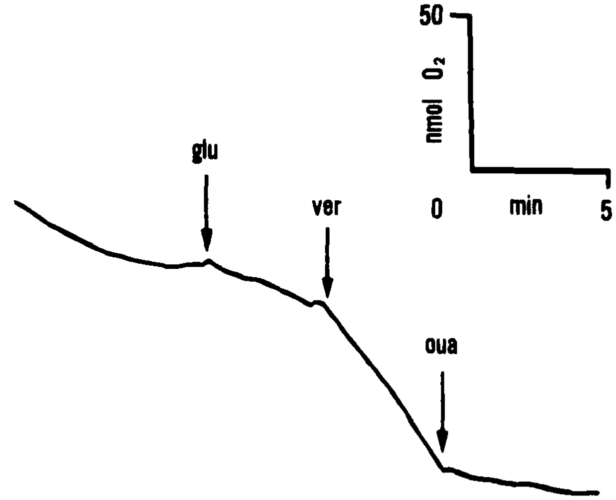

FIG. 3. Representative trace demonstrating the relationship between the rate of oxygen consumption by P_1 synaptosomes (0.32 mg protein/ml), membrane depolarization and ouabain-sensitive (Na^+-K^+)ATPase activity. Arrows indicate the times of addition of glucose (glu: 10 mM), veratridine (ver: 100 μm) and ouabain (oua: 1 mM).

P_1 fraction was derived from the P_3 fraction, competency of the P_1 synaptosomes would also indicate that the P_3 synaptosomes were functionally intact. This metabolic competency was indicated by the demonstration that these synaptosomes retained the ability to accumulate [^{3}H]-2-DG by an active, high affinity, mechanism (Fig. 2). Analysis of [^{3}H]-2-DG uptake using the standard Lineweaver-Burk format estimated the kinetic constants (K_T and V_{max}) to be 0.38 mM and 2.4 nmol/min/mg protein, respectively (Fig. 2). The intrasynaptosomal mitochondria also demonstrated respiratory control, since the respiratory rate of these synaptosomes in the presence of 10 mM D-glucose was increased from 2.9 to 11.5 nmol of O_2/min/mg protein by the addition of 100 μM veratridine (Fig. 3). Ouabain (1 mM) completely reversed the stimulatory effect of veratridine (Fig. 3). The ability of ouabain to inhibit the veratridine-induced increase in oxygen consumption suggests that this response results from the stimulation of (Na^+-K^+)-ATPase and the consequent increase in the rate of ATP utilization (31). Under the conditions of this experiment, the contaminating free mitochondria do not contribute to the observed response (31). However, our attempts to measure K^+-evoked glutamate release from the P_1 fraction yielded inconsistent results (data not shown). The P_3 fraction was consequently selected as the preparation of choice in all subsequent investigations.

Distribution of Enzymes, Zinc and Dynorphin B in Hippocampal Synaptosomal Fractions

The enzyme activities and concentrations of zinc and Dyn B-like immunoreactivity (LI) in small (P_2) and large (P_3) synaptosomal fractions isolated from hippocampal tissue are presented in Table 2. There were no significant differences in the specific activities (SA) measured for (Na^+-K^+)-ATPase or ChAT in these preparations. The P_3 fraction, however, was significantly lower in the specific activity for homogenate (ChAT SA=87.0±8.2 nmol/hr/mg protein, $p<0.01$; Student's t-test). Total LDH activity per mg protein in P_3 was significantly higher than in P_2 suggesting that the cytoarchitecture of large mossy fiber synaptosomes is relatively enriched in cytoplasm. Additional experiments confirmed that 92.5% of the total LDH activity measured in the P_3 fraction was occluded by cell membranes immediately after isolation and that greater than 88% of this activity remained occluded during 60 min of incubation at 30°C in the modified Elliott's solution described above. The specific activity of glutaminase, a reliable enzyme marker for glutamatergic nerve terminals, in the P_3 fraction was significantly lower than P_2, but higher than that measured in the homogenate (2.0±0.2 μmol/hr/mg protein, $p<0.01$; Student's t-test). Zinc and dynorphin-LI, which are considered to be useful biochemical markers for hippocampal mossy fiber terminals (11, 20, 26), were both significantly enriched in the P_3 fraction (Table 2). Zinc was enriched by nearly 2-fold in P_3 and Dyn B-LI by 4-fold relative to the smaller P_2 synaptosomes.

Release of Prodynorphin-Derived Peptides

Potassium-induced (45 mM) depolarization substantially increased the rate of release for both Dyn B-LI and Dyn A(1–8)-LI and, as reported previously for hippocampal slices (7), the rate of Dyn B-LI release was stimulated to the greatest extent. The peak release rates were 6.5±2.1 and 1.8±0.1 pg/min/mg protein for Dyn B-LI and Dyn A(1–8)-LI, respectively. These values represent a 3- to 5-fold increase over the spontaneous rates of release for these peptides.

As shown in Fig. 4, K^+-induced (45 mM) depolarization of the P_3 fraction in the absence of extracellular calcium ions (S_1) did not stimulate Dyn B-LI release. Subsequent redepolarization of these same synaptosomes in the presence of Ca^{2+} (S_2) increased the release of this peptide by a factor of 2.5 (Fig. 4).

Release of Endogenous Glutamate

Superfusates collected in the experiment described above

TABLE 2

RELATIVE ENRICHMENT OF ENZYME MARKERS, ZINC AND DYNORPHIN B IN ISOLATED RAT HIPPOCAMPAL SYNAPTOSOMES

Marker	Subcellular Fraction P_2	P_3
Lactate dehydrogenase	42.8 ± 12.6	74.4 ± 4.8*
(Na^+-K^+)-ATPase	13.6 ± 3.5	16.5 ± 4.6
Choline acetyltransferase	59.1 ± 5.6	58.1 ± 0.6
Glutaminase	3.9 ± 0.3	2.8 ± 0.1*
Zinc	109 ± 27	178 ± 7*
Dynorphin B-LI	244 ± 87	978 ± 88*

Small (P_2) and large (P_3) synaptosomes were separated and analyzed as described in the Method section. Enzyme activities are expressed as μmol/hr/mg protein. ChAT as nmol/hr/mg protein, zinc and Dyn B concentrations as pg/mg protein. Results are means ± SD from three experiments assayed in triplicate. *Significantly different ($p<0.01$; Student's t-test) from small synaptosome values.

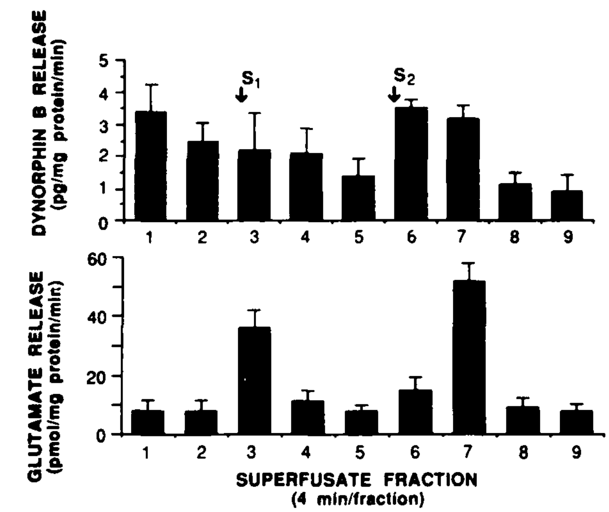

FIG. 4. Calcium-dependence of K^+-stimulated neurotransmitter release from large hippocampal synaptosomes. Top panel: Release rates for dynorphin B-LI. Bottom panel: Release rates for endogenous L-glutamate. The P_3 fraction (7 mg protein) was superfused at room temperature (25±1°C) for 8 minutes with a Ca^{2+}-free Krebs-bicarbonate medium (KBM) containing EGTA (0.5 mM). The [KCl] was then raised to 45 mM for 2 minutes (S_1) and superfused with KBM for an additional 6 minutes before removing EGTA and introducing $CaCl_2$ (1.8 mM). After 20 minutes of superfusion, the P_3 synaptosomes were depolarized once again (S_2) in an identical manner and fractions were collected for a total of 36 minutes. Results are means±SD of data (n=6) from two separate experiments.

(Fig. 4) were also assayed for their glutamate content. As reported previously (14), the K^+-evoked release of acidic amino acid neurotransmitters includes a Ca^{2+}-independent component which is thought to be contributed by the cytoplasmic pool(s) of these amino acids (27). In the present experiment, the K^+-evoked release of glutamate from P_3 was inhibited by 30% when Ca^{2+} was omitted (S_1/S_2=0.70, Fig. 4). Because the order of stimulus presentation was not varied, the depolarization-induced release of glutamate in the presence of Ca^{2+} (S_2) was always measured after a partial depletion of the releasable pool(s) and this would be expected to result in an underestimation of Ca^{2+}-dependent release.

The results shown in Fig. 5 demonstrate that addition of 50 μM D-aspartate to a nondepolarizing (5 mM KCl) superfusion medium increased the rate of spontaneous glutamate release, which is consistent with a heteroexchange of glutamate out of the cytoplasm (27), and almost completely blocked the ability of K^+-stimulation to mobilize glutamate from remaining stores in the absence of external Ca^{2+}. The Ca^{2+}-dependent component of release, however, remained virtually unchanged (Fig. 6). This finding was determined by measuring the rates of glutamate release evoked by K^+-stimulation of P_3 in the presence and absence of D-aspartate, as described previously (14). These calculations indicate that D-aspartate pretreatment reduced the total evoked release of glutamate by 37%, but had no significant effect on the size of the residual Ca^{2+}-dependent pool (Fig. 6).

DISCUSSION

Intact hippocampal mossy fiber synaptosomes, capable of expressing organized metabolic activity, can be isolated using the fractionation techniques described in this report. The large size and complex morphology of these synaptosomes are sufficiently distinctive for their identification. Biochemical analyses suggest that these synaptosomes are metabolically viable, with an active (Na^+-K^+)-ATPase system, tightly coupled intrasynaptosomal respiratory chain, a relatively uncompromised plasma membrane as indicated by the almost complete occlusion of LDH activity, and respond to membrane depolarization with a Ca^{2+}-dependent release of endogenous glutamate and dynorphin peptides. The availability of such a preparation will permit a detailed biochemical investigation of the mossy fiber synapse.

Preservation of the specialized structural characteristics of the large mossy fiber expansions requires that the tissue be manually disrupted. In attempting to adapt these techniques careful attention must be given to the homogenization conditions used. The development of this procedure was based on the methods previously reported for separation of cerebellar glomeruli (19) and guided by the results of our own electron microscopic observations. Previous attempts (11) to separate the large hippocampal mossy fiber synaptosomes have employed the homogenization conditions originally described by Israel and Whittaker (22) in which a motor-driven pestle was used for disruption of cerebellar tissue and the isolation of cerebellar glomeruli. However, cerebellar glomeruli, which are comparable in size and morphology to the hippocampal mossy fiber terminals, have been shown (19) to be disrupted by these conditions. In the present studies even minor modifications of the more gentle manual techniques used for tissue disruption could noticeably alter the appearance of the resulting subcellular particles.

The isolation of a subcellular fraction that is clearly enriched in hippocampal mossy fiber synaptosomes, regardless of the degree to which it has been purified, would only constitute a significant advance if these presynaptic terminals retain their functional integrity. The P_4 fraction was found to be enriched, relative to P_3, in mossy fiber synaptosomes and retained a considerable degree of glycolytic and respiratory control. The cytological characteristics of the P_4 synaptosomes indicated that a significant loss of synaptic vesicles had occurred, however, and the results of prelimi-

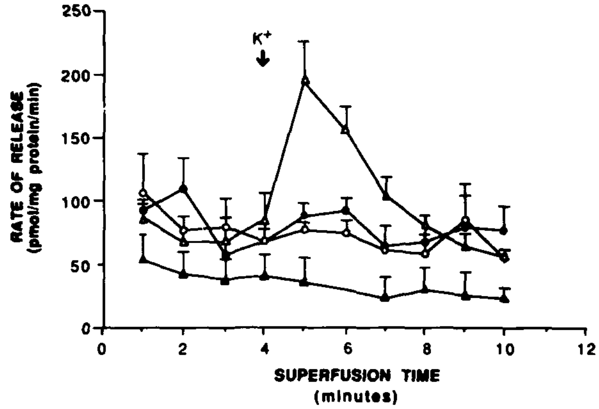

FIG. 5. Effect of D-aspartate on the spontaneous and K^+-stimulated release of endogenous L-glutamate from P_3 synaptosomes. The P_3 pellet was resuspended in a Ca^{2+}-free Elliott's medium to a final concentration of 15 mg protein/ml and incubated for 5 minutes at 30°C. Aliquots (250 μl) of this suspension were transferred to parallel glass superfusion chambers, thermostatted to 30°C, and superfused for 5 minutes with a low K^+ (3.1 mM) medium containing $CaCl_2$ (1.8 mM). One minute fractions were then collected and assayed for glutamic acid (see the Method section). Treatment conditions were as follows: (▲) low K^+ medium; (○) low K^+ medium with D-aspartate (50 μm) added; (●) Ca^{2+}-free medium containing D-aspartate, [KCl] raised to 45 mM at arrow for 2 minutes; (△) same as (●), except $CaCl_2$ present. Results are means±SD of duplicate determinations from three separate experiments.

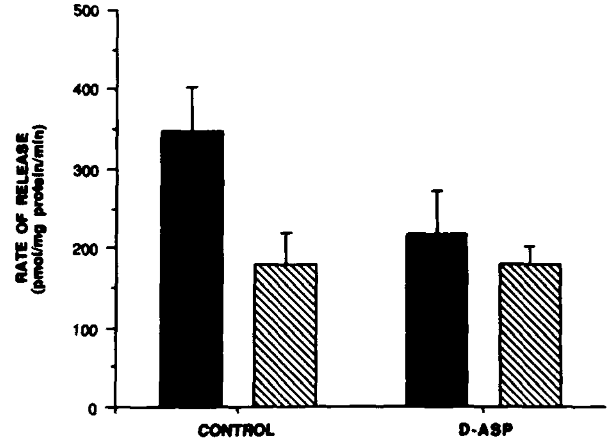

FIG. 6. Effect of D-aspartate (D-ASP) on total evoked L-glutamate release and its Ca^{2+}-dependent component. The rates of glutamate release during the initial 3 minutes of 45 mM K^+-stimulation were integrated and expressed as the means±the standard errors of the difference between means of duplicates from three separate experiments. Total release (solid bars) evoked in the presence of $CaCl_2$ (1.8 mM) was measured with (D-ASP) or without (Control) D-aspartate pretreatment. The Ca^{2+}- dependent component (cross-hatched bars) of release was determined by stimulating P_3 synaptosomes, in parallel superfusion chambers, with a Ca^{2+}-free medium containing equimolar KCl.

nary glutamate release studies were inconsistent. The P_3 fraction was, therefore, selected for further investigations in which this synaptosomal preparation is highly responsive to K^+-induced depolarization and that amino acid neurotransmitter pools remained compartmentalized within these synaptosomes.

Electron microscopic examination showed that all of the larger synaptosomes (2 μm diameter or greater) in the P_3 fraction were easily identifiable as mossy fiber terminals. Each synaptic profile had a complex irregular outline, exhibited both symmetric and asymmetric synaptic contacts, and contained one or more invaginated dendritic spines (Fig. 1B). In addition, each contained numerous clear synaptic vesicles, several mitochondria and a few dense core vesicles. All of these characteristics have been reported for mature mossy fiber terminals in vivo (2). The isolated mossy fiber synaptosomes examined here, however, were found to be smaller than in vivo mossy fiber terminals. For example, the average perimeter of 18 selected P_3 mossy fiber terminals was approximately 11 μm (see Table 1), whereas Amaral and Dent (2) reported an average perimeter of 20 μm for mature mossy terminals in vivo. There are several possible reasons for the smaller size of the isolated terminals. First, some of the isolated P_3 synaptosomes were most likely axon collateral terminals from the dentate hilar region. These terminals have the same unique morphology as the mossy fiber terminals present in the hippocampus, but are smaller—between 2 and 4 μm in diameter (8). Second, although the synaptosomes in the homogenate and fractions appeared intact, there was undoubtably some disruption during the isolation procedures which, after resealing of the plasma membrane, resulted in large fragments of the mossy fiber expansion. Lastly, no attempt was made here to correct for either sectioning biases, or for fixation-induced shrinkage, which is likely to be greater for isolated terminals than for perfused brain tissue.

In addition to the large mossy fiber terminals, numerous synaptosomes averaging slightly less than 1 μm in diameter were present in both the P_3 and the P_4 fractions. Using a modification of the Timm's stain that we developed for isolated nerve terminals (manuscript in preparation), we recently found that approximately one-third of the smaller synaptosomes in the P_4 fraction stain for zinc (as do all of the larger mossy fiber synaptosomes). These results suggest that at least some of the smaller synaptosomes are either pieces of mossy fiber terminals or are synaptic terminals from the dentate molecular layer and hippocampal commissural zones that are known to stain with the Timm's technique in sections of the intact hippocampus. We are presently determining what proportion of the smaller synaptosomes in the P_3 fraction are zinc-containing.

Measurements of endogenous zinc (Table 2) give much the same result, indicating that zinc is enriched 2-fold in the P_3 fraction compared to P_2. As mentioned above, modification of the Timm's staining procedure shows that all isolated mossy fiber synaptosomes and some small synaptosomes contain zinc (manuscript in preparation). Dyn B-LI was also found to be enriched in the P_3 synaptosomal preparation (Table 2) and is released in a Ca^{2+}-dependent manner by K^+-induced depolarization. These findings are consistent with the observations that both zinc and the prodynorphin-derived peptides are concentrated in the mossy fiber terminals in vivo (9,26) and clearly support the suggestion that the P_3 fraction is enriched in mossy fiber synaptosomes.

Membrane depolarization stimulated Ca^{2+}-dependent release of endogenous glutamate and two different prodynorphin-derived peptides. The present results confirm a previous report that, of these opioids, the release rate for Dyn

B-LI is stimulated to the greatest extent (7). To examine the Ca^{2+}-dependence of K^+-evoked neurotransmitter release in this preparation, the membranes were twice exposed to 45 mM KCl, first in the absence of $CaCl_2$ and subsequently in the presence of 1.8 mM $CaCl_2$ (Fig. 4). The data show that: 1) the failure of the first pulse of high [KCl] to stimulate Dyn B-LI is not due to a depleted endogenous pool of releasable Dyn B since the second pulse did evoke release; 2) the release of this opioid peptide has a strict requirement for extracellular Ca^{2+}; and 3) the K^+-stimulated release of endogenous glutamate is only partially dependent on the presence of Ca^{2+}. This provides convincing evidence that the K^+-evoked release of Dyn B from this subcellular fraction is mediated by a Ca^{2+}-dependent mechanism. However, our observations regarding the Ca^{2+}-dependence of glutamate release were less conclusive and led us to a more careful examination of the mechanism of this K^+-evoked efflux.

D-aspartate (50 μM) freely exchanges with the cytoplasmic pool of endogenous glutamate in nerve terminals, but only slowly gains access to the vesicular compartment (27). Glia also accumulate D-aspartate (5,33), a glutamate analogue that is not metabolized by glial cells to any appreciable extent. However, glutamate itself is not stored by glia but rapidly degraded and the metabolites are released (33). Therefore, that glutamate which is not displaced by D-aspartate is presumably stored in a noncytoplasmic compartment of presynaptic nerve terminals and is released in a Ca^{2+}-dependent fashion by membrane depolarization (Fig. 5). This finding confirms previous reports (27,29) that the Ca^{2+}-dependent and -independent pools of K^+-evoked glutamate release can be dissociated in this fashion and can be generalized to other species and brain regions. Of perhaps greater interest, however, is the demonstration by these experiments that, once dissociated by D-aspartate exchange, there is little if any replenishment of the Ca^{2+}-independent glutamate pool within the time course of these experiments. It is possible, therefore, to adapt the use of this powerful technique to standard superfusion systems for investigating the mechanism of glutamate exocytosis and its modulation. Using the same basic principle, heteroexchange of an amino acid transmitter out of the cytoplasm, it is also possible to isolate the exocytotic pool of gamma-aminobutyric acid (35).

In conclusion, the subcellular fractionation approach taken in these experiments provides a preparation suitable for biochemical studies of the presynaptic mechanisms involved in hippocampal mossy fiber synaptic transmission. Such a preparation will be useful in identifying the factors that modulate the release of amino acid and opioid neurotransmitters from these specialized structures and in the deduction of their presynaptic mechanisms of action.

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